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Most high-grade neuroendocrine tumours of the lung are likely to secondarily develop from pre-existing carcinoids: innovative findings skipping the current pathogenesis paradigm

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Most high-grade neuroendocrine tumours of the lung develop secondarily from preexisting carcinoids: Unexpected findings skipping the current pathogenesis paradigm

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ABSTRACT (210 words)

Lung neuroendocrine tumours (Lung-NETs) include typical carcinoid (TC), atypical carcinoid (AC), large cell neuroendocrine carcinoma (LCNEC) and small cell lung carcinoma (SCLC). On molecular grounds, TC and AC are considered separate tumour entities as opposed to LCNEC and SCLC.

By means of two-way clustering analysis of previously reported next generation sequencing data on 148 surgically resected Lung-NETs, we identified six different clusters (C1 → C6) accounting for 68% of tumours, where specific sets of molecular alterations were embedded within different histologic subtypes. The clustering tree organization suggested the low-grade Lung-NETs may evolve into high-grade Lung-NETs following two paths: C5→C1→C6 and C4→C3-C2. The tumour composition of the first path (C5→C1→C6) would be coherent with the hypothesis of an evolution of TC to LCNEC, even with a conversion of some SCLC to LCNEC. The second group (C4→C3-C2) has a tumour composition supporting the hypothesis of evolving AC to SCLC-featuring tumours. Interestingly, the Ki-67 labelling index varied accordingly in a significant way, with median values being 5%, 9% and 50% in cluster C5, C1 and C6 and 12% and 50-60% in cluster C4 and C2-C3, respectively.

This is a proof of concept study supporting an innovative view on the progression to high-grade NE carcinomas of preexisting TC or AC in most Lung-NET instances.

Key Words: lung; neuroendocrine; tumours; cluster analysis; transition; secondary

INTRODUCTION

Lung neuroendocrine tumours (Lung-NETs) are currently divided into four histologic variants according to necrosis amount, number of mitoses per 2 mm² and a wide constellation of cytological and histological traits [1,2]. They include typical carcinoid (TC), atypical carcinoid (AC), large-cell neuroendocrine carcinoma (LCNEC) and small-cell carcinoma (SCC) [1,2]. This pathologic classification fits with a three-tier clinical scheme, according to which TC behave as low-grade malignant tumours, AC as intermediate-grade malignant tumours and the group of LCNEC and SCLC as high-grade malignant tumours with no significant survival differences [1,2].

The current believes support the notion that TC and AC make up different and separate tumour entities as opposed to LCNEC and SCLC [1,3-10]. Nonetheless, carcinoids and NE carcinomas may share several genetic alterations, yet with different prevalence rates, which push an unexpected concept of secondary evolution to be hypothesized in NETs of different organs [7,11-20]. This bewildering situation, which does not comply with the current classification scheme [1], has been giving a variety of terms, such as *i)* high-grade NE carcinoma with carcinoid morphology [21], *ii)* secondary high-grade NET [11], *iii)* well differentiated NET with a morphologically apparent high-grade component [13], *iv)* transformed or mixed grade NET [13], *v)* well differentiated NET with high-grade (G3) progression [22], *vi)* carcinoid-like LCNEC with MEN1 mutation [8,23], *vii)* carcinoid or NET with proliferation rate progression at metastatic sites [24,25], *viii)* progression of pulmonary carcinoid tumours [17-20], *ix)* NE carcinoma with combined features ranging from well-differentiated (carcinoid) to small cell carcinoma [26] and *x)* the recently introduced category of G3 NET [27,28]. The relative scarce knowledge on Lung-NET biology certainly limits our comprehension regarding the real origin of these tumour subtypes, which have been rather considered exceptions or outliers to the current pathogenesis models of separate tumour derivation [1,28]. However, there is also the possibility for carcinoids to progress towards high-grade NETs as a result of either the natural history of the disease or the pressure of therapies [11]. This would represent an alternative view underlying the development of most NETs arising anywhere in the body [29-31]. It is worth mentioning that these secondary NETs show a better prognosis and a different clinical presentation in comparison with *de novo* or primary high-grade NETs [11,13], and that they may share common molecular traits and cancer drivers with carcinoids [11,13,14,16,32].

We herein provide a molecular classification of a large cohort of 148 Lung-NETs comprising all histologic variants. Our findings support the hypothesis that aggressive Lung-NETs could evolve from different histologic subtypes after crucial gene alterations have been acquired during tumour progression.

PATIENTS AND METHODS

Patients and tumours

This study comprises a cohort of 148 Lung-NETs belonging to all histologic variants, which had previously been investigated by next generation sequencing (NGS) [12]. Gene alteration results, including either mutations or copy number variations (CNVs), have been analysed to test the hypothesis of secondary Lung-NETs developing from pre-existing carcinoids. The cohort comprises 53 TC, 35 AC, 27 LCNEC e 33 SCLC according to the current World Health Organization (WHO) 2015 classification [1,2], all surgically treated and with no neoadjuvant treatments. The original material had been assayed in a discovery set by whole exome sequencing and high-coverage targeted sequencing and then validated by either customized or commercially available multi-gene panels for recurrent mutations and CNVs [12]. Paraffin tumour specimens had also been assessed by immunohistochemistry (IHC) for the expression of Ki-67 antigen [12].

Ethics

As this study dealt with reanalysis of previously generated and authorized molecular data by local Ethics Committees [12], no further ethics release was necessary.

Study design

This is an *in silico* study generating a proof of concept concerning an innovative concept on secondary Lung-NETs developing through sequential gene alterations accounting for mutations and CNVs. We previously generated a list of 89 recurrently altered genes in Lung-NETs [12], from which 40 unbiased recurrent gene alterations present in at least three different tumours were abstracted to guarantee specificity to molecular findings by minimizing the role of hitchhiking gene alterations. This 40-gene signature included mutations in 27 genes and CNVs in 13 genes. The 27 genes harbouring recurrent mutations were *ARID1A*, *ARID1B*, *ARID2*, *ATM*, *CSMD3*, *DSCAML1*, *DSCAML1*, *KMT2C/MLL3*, *KMT2D/MLL2*, *KRAS*, *LRP1B*, *MEN1*, *NCAM2*, *NOTCH2*, *PBRM1*, *PCLO*, *PIK3CA*, *PTPRZ1*, *RB1*, *SETD2*, *SMARCA2*, *SMARCA4*, *SPHKAP*, *STK11*, *THSD7B*, *TP53*, *TSC2*. The 13 genes tested for CNVs were *BCL2*, *FGFR1*, *MEN1*, *MYC*, *MYCL*, *PIK3CA*, *RICTOR*, *RB1*, *SDHA*, *SMAD4*, *SRC*, *TERT*, *TP53*, as previously detailed [12]. Such a 40-gene signature was thus used for tumour analysis by clustering evaluation.

Statistical analysis

Hierarchical clustering analysis was performed using Cluster 3.0 software (<http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm>) and JAVA treeview (<http://jtreeview.sourceforge.net>) on categorized data. This categorization ranked 0 for wild type configuration (WT), 1 for mutation or loss of heterozygosity (LOH) or low-gain (3-5 copies); and 2 for deletion or high-gain (> 5 copies), as also explained in the Figure 1A legend. Clustering metrics used were Spearman rank correlation and average linkage. Frequencies of genetic alteration were calculated by averaging the number of altered samples in each cluster. Hierarchical clustering analysis of frequencies was done using un-centered correlation and average linkage. Bar plots and radar plots were prepared using Excel 2015 (Microsoft). Kaplan-Meier plots and log-rank statistical analysis was performed using JMP 12 (SAS). Univariate and multivariate analysis was performed using the SAS statistical software, version 9.2 (SAS Institute, Inc., Cary, NC). Categorical variables were compared by Kruskal-Wallis test, Fisher exact t test and chi-square test as appropriate. All p-values were two-sided and p-values <0.05 were considered as significant.

RESULTS

Hierarchical clustering analysis reveals distinct groups of tumours

We initially performed supervised clustering analysis to evaluate the distribution of gene alterations across our cohort of 148 Lung-NETs according to histologic subtyping. One-way clustering analysis confirmed that TC showed the lowest burden of gene alterations, while AC, LCNEC and SCLC progressively accumulated gene mutations and CNVs (**Supplemental Figure 1A** and **Supplemental Table 1**). The clinico-pathologic characteristics of the tumour series according to histologic subtyping are reported in **Supplemental Table 2**. Survival curves confirmed an excellent prognosis for TC, an intermediate prognosis for AC and a progressively deteriorated clinical course for LCNEC and SCLC (**Supplemental Figure 1B**).

We next performed unsupervised clustering analysis using the same 40-genes set. This analysis uncovered six distinct clusters, named C1→C6, which showed different patterns of gene alterations, either mutations or CNVs (**Figure 1A**), suggesting the existence of molecular subtypes which included different histologic subtypes. These clusters accounted for 100 (68%) of the 148 Lung-NETs, while the remaining unclassifiable tumours were descriptively labelled as “unclassified”. The C1→C6 clusters were characterized by one or more gene alterations with a prevalence higher than 0.5 (i.e. 50% of samples) in the relevant tumour clusters (**Figure 1B**). They included *i*) *RB1* deletion (*RB1-del*) in cluster C1, *ii*) *TP53* mutation and deletion (*TP53-mut* and *TP53-del*), *RICTOR/SDHA/TERT* copy number gains (*RICTOR-gain*, *SDHA-gain* and *TERT-gain*) in cluster C2, *iii*) *TP53* mutation (*TP53-mut*) and *RICTOR* copy number gain (*RICTOR-gain*) in cluster C3, *iv*) *TERT* and *SDHA* gain (*TERT-gain* and *SDHA-gain*) in cluster C4, *v*) *TP53* deletion (*TP53-del*) and *MYC* gain (*MYC-gain*) in cluster C5, and *vi*) *KRAS/TP53* mutation (*KRAS-mut* and *TP53-mut*) and *MYCL* copy number gain (*MYCL-gain*) in cluster C6 (**Figure 1B**).

To further investigate the relationship among C1→C6 clusters, we extended two-way clustering analysis considering the prevalence of alterations of all genes analysed (N=40) (**Figure 1C**). Notably, C5 cluster closely resembled clusters C1 (**Figure 1C**) for sharing *TP53/RB1/MEN1* gene deletion along with *MYC* and *PIK3CA* gains (**Figure 2A**). In turn, C6 cluster was similar to C1 for *MEN1/RB1/TP53* deletion, *KMTD2/TP53* mutation, and *MYC* gain (**Figure 2A**, upper-middle part). Furthermore, C4 cluster shared with C6 *MYC/MYCL/RICTOR* gains, *RB1/TP53* deletion and *MEN1/KMT2D-MLL2/TP53* mutation (**Figure 2A**, upper-right part) while *KRAS* mutation was private to

cluster C6 (**Figure 2A**). Finally, C3 cluster showed an expansion of *RICTOR* gain and *TP53* mutation (**Figure 2A**, lower-left part) which was maintained in C2 cluster like all other genetic alterations identified, whereas *MEN1* deletion was private to C4 cluster. However, C2 cluster was further characterized by an evident expansion of *RB1/TP53* deletion, *TERT/SDHA* gains and, marginally, *LRP1B* mutation (**Figure 2A**, lower-right part). Therefore, most tumours showed high levels of shared mutations despite different tumour subtyping in the identified clusters (**Figure 2B**), thus suggesting a common evolutionary relationship. Interestingly, the median Ki-67 LI was 5%, 9% and 50% in clusters C5, C1 and C6 and 12% and 50-60% in clusters C4 and C2-C3, respectively (**Table 1**). Furthermore, Ki-67 immunopositivity was heterogeneously distributed in neoplastic cells at the level of individual high-grade tumours (**Supplemental Figure 2**).

Univariate and multivariate analysis

Clusters differed statistically for gender, smoking status, histological subtyping and Ki-67 positivity, but not for age of patients and tumour stage (**Table 1**). Of note, a large prevalence of carcinoids (26/31 tumours, 84%) but with inverted frequency was observed in cluster C5 (9 TC and 3 AC out of 14 tumours) and C4 (2 TC and 12 AC out of 17 tumours), whereas the opposite held true for high-grade Lung-NETs (27/35 tumours, 77%), which prevailed in cluster C3 (6 LCNEC and 4 SCLC out of 15 tumours) and cluster C2 (4 LCNEC and 13 SCLC out of 20 tumours) (**Figure 2B**; **Table 1**). Lack of SCLC was notable in cluster C6 where there was a slight prevalence of LCNEC (4/7 tumours) over TC and AC (**Figure 2B**; **Table 1**), while AC were virtually absent in cluster C1 in front of prevalent TC, SCLC and LCNEC (**Figure 2B**; **Table 1**). Unclassified tumours were 48 and included mostly carcinoids (39/48 cases, 81%), especially TC (26 cases), while high-grade NETs (4 LCNEC and 5 SCLC) were modestly represented (**Figure 2B**, **Table 1**).

Cox univariate analysis showed that age, male gender, smoking habit, tumour stage, histologic subtyping and clusters affected survival (**Table 2**). Multivariate analysis confirmed that age, histological subtyping (SCLC), tumour stage (III-IV), and C3 affected survival, independently of other clinical-pathological parameters (**Table 2**).

DISCUSSION

Our results support a paradigm shift to the current view of the pathogenesis of Lung-NETs by suggesting that TC have a potential to evolve in high-grade tumours, either directly or through intermediate steps, resembling the more aggressive LCNET and SCLC subtypes in terms of acquired molecular alterations. These findings confirm the concept of secondary NETs we recently authorised in NETs arising in the thymus [11]. A plethora of different terms has been used in NET literature to describe these secondary tumours arising in different organs [8,11,13,17-21,23-26,33,34]. The recently suggested category of NET G3 in the gastrointestinal tract [6,27], which has been now introduced in the 2017 WHO classification of endocrine tumours [28], may well represent secondary NETs arising from pre-existing G1/G2 NETs. It is tempting to hypothesise that this peculiar phenomenon is inherent to NETs at different sites and not so rare as currently believed [17-20]. We herein provide molecular demonstration that most Lung-NETs may belong to this category, and are possibly not unpredictable exceptions or bewildering outliers stochastically crossing the spectrum of NETs [1,3-9], but rather a systematic biologic phenomenon, thus realizing an innovative view in the development of Lung-NETs.

Our starting observation was that carcinoids and NE carcinomas in different organs may share several genetic abnormalities, yet with different prevalence rates, such as mutations, CNVs and microRNA expression levels [7,11-16]. Moreover, the existence of patient subsets with different life expectation in each histologic variant of Lung-NETs is in keeping with an evolution concept within the clinico-pathologic spectrum of these tumours [17-20,35]. In this scenario, we underwent an *in silico* analysis on our previously published molecular data [12], which confirmed the existence of distinct clusters of tumours with a heterogeneous composition of histologic subtypes showing several common genomic abnormalities. Additional sources for validation, such as Cancer Genome Atlas (<https://cancergenome.nih.gov>) or NGS studies on carcinoids [3], LCNEC [8,9] or SCLC [4], were discharged due to lack of comparable information on histologic subtypes, survival or mutation/CNV details.

Our study provides new molecular evidences to the existence and prevalence of secondary NETs in the lung. These tumours, which have not been comprised in the 2015 WHO classification [1,2], share molecular alterations indicative of a common origin with additional gene alterations likely occurring over time, which would be instrumental to the development of high-grade tumours [11]. The

opposite situation, *i.e.* the down-grading of poorly differentiated NETs, is contradicted by the additional gene aberrations occurring in high-grade elements [11] and the generally less aggressive clinical behaviour attributed to these tumours [11,13,25]. Of note, secondary-type evolution would not be confined to NETs, but can be observed in glioblastoma evolving from long-standing astrocytoma [36,37] or triple negative breast cancer stemming from adenoid cystic carcinoma [38].

As morphology alone may be deceptive and inconclusive, the existence of secondary NETs has been questioned, with only single case reports or small tumour series being described in NET literature [8,11,13,17-21,23-26,33,34]. We herein provide the first molecular evidence that secondary NETs are under-recognized entities in the lung inasmuch as they would represent the majority (in our hands, 68%) of tumours arising in this organ. It is worth noting that in many Lung-NET series about 20-30% of high-grade tumours fulfilling criteria for either SCLC or LCNEC showed long survival or presented with organoid NE architecture featuring trabeculae, ribbons and palisading aggregates [35,39-42]. This observation could imply that high-grade NETs investigated on surgical specimens (including many of those thus far published in NGS studies) could represent secondary tumours according to our prevalence rate results compared to homologous tumours presenting at onset with metastatic disease and thus unsuitable for surgical resection. The uneven distribution of Ki-67 immunostaining within tumours, as highlighted by the differential distribution of Ki-67 among clusters we documented also at the level of individual tumours, could help to better highlight double components of low- and high-grade cells within secondary NETs, as demonstrated in the thymus [11] and the pancreas [13].

In our study, six clusters could be hierarchically organized according to unsupervised analysis, with the diverse tumour subtypes differentially distributed among them as a function of different evolution lines not predicted upon morphology grounds. On the basis of the clustering tree organization, two evolutionary related groups could be identified: C5→C1→C6 and C4→C3-C2. The first group showed differential inactivation of tumour suppressor gene upon deletion or mutation alongside with *MYC* copy number gains and private *KRAS* mutation limited to cluster C6 in a clinical setting of predominantly male patients with smoking habit. The different tumour composition of these clusters would be coherent with the hypothesis of an evolution of TC to LCNEC, even with a conversion of some SCLC to LCNEC. The recently described categories of carcinoids with proliferation rate progression at metastatic sites [24], carcinoid-like LCNEC [8,11], NSCLC-like LCNEC

harbouring *KRAS* mutation [8,9] and the well-known low diagnostic reproducibility of LCNEC diagnosis towards either AC or SCLC [43,44] support all our observations. The second group showed differential copy number gains at several genes (*TERT*, *SDHA*, *MYCL*, *RICTOR*) alongside *TP53* inactivation upon deletion or mutation and *RB1* deletion, with private *MEN1* deletion in C4 where AC prevailed [17], in a clinical setting of predominantly male patients with smoking habit. The tumour composition of these clusters would be in keeping with the evolution of AC to SCLC-featuring tumours or SCLC-like LCNEC as described by others [8]. Interestingly, the Ki-67 immunostaining varied accordingly in a significant way, with median values being 5-9% in clusters C1-C5 and 50-60% in clusters C2-C3. All these observations indicated that some TC or AC harbouring a particular combination of smoke-related genetic alterations and/or male gender had the potential to give rise to high-grade Lung-NETs. The description of metastatic pulmonary carcinoid tumours [24] or pancreatic NETs [6,25] showing proliferation rate progression at metastatic sites and featuring SCLC-like [24] or NET G3-like [25] appearance, are in keeping with our results.

Limitations to our study were its retrospective character, the small number of tumours in each cluster, the partial follow-up, the absence of independent validation cohorts and the lack of cell lines or murine models replicating these evolution lines. However, molecular data by others [8] on LCNEC originally classified as NSCLC-like LCNEC and SCLC-like LCNEC according to major molecular profiles could be re-grouped into three different clusters where molecular subtyping was partly admixed, which identified an evolution plasticity in accordance with our clustering results (**Supplemental Figure 3**). Recent data on primary and secondary SCLC and LCNEC developing from NSCLC with NE differentiation according to somewhat similar molecular alterations [33] further confirm our observations. Therefore, these secondary NETs, while realizing a paradigm shift to the current pathogenesis, are an integral part of the natural history of NETs, either spontaneous [11] or resulting from therapy pressure [45].

CONCLUSION

This *in silico* study of mutations and CNVs identified widely shared alterations common to the majority of Lung-NETS, thus supporting a concept of evolution of carcinoids towards high-grade tumours. The clinical implications and the precise molecular mechanisms of these secondary Lung-NETs will have to be deepened in the near future by additional studies.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

TABLE LEGENDS

Table 1. Clinical and pathological characteristics by clustering analysis

Table 2. Univariate and multivariate survival analysis

FIGURE LEGENDS

Figure 1. Molecular analysis of Lung-NETs. **A.** Unsupervised hierarchical cluster analysis of the 40-gene alterations identified in the 148 samples cohort. Six clusters were identified, named C1→C6, and accounted for 100 out of 148 tumour samples while the remaining 48 remained unclassified with the gene signature under evaluation. Types of genetic alterations are explained by using different colours, as per the legend. “Amp” stands for amplification/gain, “del” for deletion/loss, “mut” for mutation, “LOH” for loss of heterozygosity and “Homo” for homozygous deletion. Low and high amplification/gain was defined by the cut-off threshold of 5 as previously detailed (see reference #12). **B.** Radar plot of prevalent genetic alterations (frequency>0.5, i.e. 50%) in the clusters identified in Figure 1A. Numbers identify the percentages of the relevant gene alterations. **C.** Hierarchical cluster analysis of frequencies of all genetic alterations found in C1→C6 clusters. Only prevalent genetic alterations (frequency>0.5) are shown.

Figure 2. **A.** Genetic alterations shared by various clusters (C1→C6). Bar plots indicate frequency (Y-axes) of the alterations found in each cluster (shown on X-axes). Asterisks indicate significant p-values calculated by chi-square test. **B.** Distribution of the four World Health Organization histologic subtypes in the various clusters identified in Figure 1A. The pie charts indicate distribution of different tumour types in the clusters and were positioned according to the clustering tree shown in Figure 1C. TC stands for typical carcinoid, AC for atypical carcinoid, LCNEC for large cell neuroendocrine carcinoma and SCLC for small cell lung carcinoma, while unclassified groups the remaining 48 Lung-NETs not entering clusters C1→C6.

SUPPLEMENTAL FILES

Supplemental Table 1. Mean gene alterations per tumour according to histologic subtyping

Supplemental Table 2. Clinical and pathologic data of 148 neuroendocrine tumours of the lung according to histology

Supplemental Figure 1. A. Supervised clustering analysis of gene alterations across the entire spectrum of Lung-NETs. Different tumours and types of genetic alterations are colour coded as per the legend. TC stands for typical carcinoid, AC for atypical carcinoid, LCNEC for large cell neuroendocrine carcinoma and SCLC for small cell lung carcinoma. **B.** Survival analysis by Kaplan-Meier plot of tumours stratified by histologic subtyping.

Supplemental Figure 2. High-grade NET consistent with SCLC in the cluster C1 (**A**) showed an intra-tumour compartmentalization of the Ki-67 immunostaining with an intimate admixture of proliferating and non-proliferating tumour cells within the same tumour case. This distribution of tumour cells ruled out a collision tumour and was in agreement with the evolution of a preexisting carcinoid to high-grade SCLC-like NET (**B**).

Supplemental Figure 3. Unsupervised cluster analysis on the data set published by Rekhtman et al (see reference #8), regarding NGS analysis of a large series of LCNEC of the lung.

REFERENCES

1. Travis W, Brambilla E, Burke A, *et al.* WHO Classification of Tumours of the Lung, Pleura, Thymus and Heart. (Fourth Ed. ed). IARC Press: Lyon, 2015.
2. Travis WD, Brambilla E, Nicholson AG, *et al.* The 2015 World Health Organization Classification of Lung Tumors: Impact of Genetic, Clinical and Radiologic Advances Since the 2004 Classification. *J Thorac Oncol* 2015; **10**: 1243-1260.
3. Fernandez-Cuesta L, Peifer M, Lu X, *et al.* Frequent mutations in chromatin-remodelling genes in pulmonary carcinoids. *Nat Commun* 2014; **5**: 3518.
4. Peifer M, Fernandez-Cuesta L, Sos ML, *et al.* Integrative genome analyses identify key somatic driver mutations of small-cell lung cancer. *Nat Genet* 2012; **44**: 1104-1110.
5. Pleasance ED, Stephens PJ, O'Meara S, *et al.* A small-cell lung cancer genome with complex signatures of tobacco exposure. *Nature* 2010; **463**: 184-190.
6. Scarpa A, Chang DK, Nones K, *et al.* Whole-genome landscape of pancreatic neuroendocrine tumours. *Nature* 2017; **543**: 65-71.
7. Strobel P, Zettl A, Shilo K, *et al.* Tumor genetics and survival of thymic neuroendocrine neoplasms: a multi-institutional clinicopathologic study. *Genes Chromosomes Cancer* 2014; **53**: 738-749.
8. Rekhtman N, Pietanza MC, Hellmann MD, *et al.* Next-Generation Sequencing of Pulmonary Large Cell Neuroendocrine Carcinoma Reveals Small Cell Carcinoma-like and Non-Small Cell Carcinoma-like Subsets. *Clin Cancer Res* 2016; **22**: 3618-3629.
9. Miyoshi T, Umemura S, Matsumura Y, *et al.* Genomic Profiling of Large-Cell Neuroendocrine Carcinoma of the Lung. *Clin Cancer Res* 2017; **23**: 757-765.
10. Swarts DR, Ramaekers FC, Speel EJ. Molecular and cellular biology of neuroendocrine lung tumors: Evidence for separate biological entities. *Biochim Biophys Acta* 2012; **1826**: 255-271.
11. Fabbri A, Cossa M, Sonzogni A, *et al.* Thymus neuroendocrine tumors with CTNNB1 gene mutations, disarrayed ss-catenin expression, and dual intra-tumor Ki-67 labeling index compartmentalization challenge the concept of secondary high-grade neuroendocrine tumor: a paradigm shift. *Virchows Arch* 2017.

12. Simbolo M, Mafficini A, Sikora KO, *et al.* Lung neuroendocrine tumours: deep sequencing of the four World Health Organization histotypes reveals chromatin-remodelling genes as major players and a prognostic role for TERT, RB1, MEN1 and KMT2D. *J Pathol* 2017; **241**: 488-500.
13. Tang LH, Untch BR, Reidy DL, *et al.* Well-Differentiated Neuroendocrine Tumors with a Morphologically Apparent High-Grade Component: A Pathway Distinct from Poorly Differentiated Neuroendocrine Carcinomas. *Clin Cancer Res* 2016; **22**: 1011-1017.
14. Vijayvergia N, Boland PM, Handorf E, *et al.* Molecular profiling of neuroendocrine malignancies to identify prognostic and therapeutic markers: a Fox Chase Cancer Center Pilot Study. *Br J Cancer* 2016; **115**: 564-570.
15. Vollbrecht C, Werner R, Walter RF, *et al.* Mutational analysis of pulmonary tumours with neuroendocrine features using targeted massive parallel sequencing: a comparison of a neglected tumour group. *Br J Cancer* 2015; **113**: 1704-1711.
16. Voortman J, Lee JH, Killian JK, *et al.* Array comparative genomic hybridization-based characterization of genetic alterations in pulmonary neuroendocrine tumors. *Proc Natl Acad Sci U S A* 2010; **107**: 13040-13045.
17. Swarts DR, Claessen SM, Jonkers YM, *et al.* Deletions of 11q22.3-q25 are associated with atypical lung carcinoids and poor clinical outcome. *Am J Pathol* 2011; **179**: 1129-1137.
18. Swarts DR, Henfling ME, Van Neste L, *et al.* CD44 and OTP are strong prognostic markers for pulmonary carcinoids. *Clin Cancer Res* 2013; **19**: 2197-2207.
19. Swarts DR, Scarpa A, Corbo V, *et al.* MEN1 Gene Mutation and Reduced Expression Are Associated With Poor Prognosis in Pulmonary Carcinoids. *J Clin Endocrinol Metab* 2014; **99**: E374-378.
20. Swarts DR, Van Neste L, Henfling ME, *et al.* An exploration of pathways involved in lung carcinoid progression using gene expression profiling. *Carcinogenesis* 2013; **34**: 2726-2737.
21. Quinn AM, Chaturvedi A, Nonaka D. High-grade Neuroendocrine Carcinoma of the Lung With Carcinoid Morphology: A Study of 12 Cases. *Am J Surg Pathol* 2017; **41**: 263-270.
22. Tang LH, Basturk O, Sue JJ, *et al.* A Practical Approach to the Classification of WHO Grade 3 (G3) Well-differentiated Neuroendocrine Tumor (WD-NET) and Poorly Differentiated Neuroendocrine Carcinoma (PD-NEC) of the Pancreas. *Am J Surg Pathol* 2016.

23. Debelenko LV, Swalwell JI, Kelley MJ, *et al.* MEN1 gene mutation analysis of high-grade neuroendocrine lung carcinoma. *Genes Chromosomes Cancer* 2000; **28**: 58-65.
24. Desmeules P, Sabari J, Santos-Zabala ML, *et al.* Metastatic lung carcinoid tumors: evidence of proliferation rate progression at metastatic sites (Abstract #1911). *Mod Pathol* 2017; **30**: 475A.
25. Pelosi G, Bresaola E, Bogina G, *et al.* Endocrine tumors of the pancreas: Ki-67 immunoreactivity on paraffin sections is an independent predictor for malignancy: a comparative study with proliferating-cell nuclear antigen and progesterone receptor protein immunostaining, mitotic index, and other clinicopathologic variables. *Hum Pathol* 1996; **27**: 1124-1134.
26. Moran CA, Suster S. Thymic neuroendocrine carcinomas with combined features ranging from well-differentiated (carcinoid) to small cell carcinoma. A clinicopathologic and immunohistochemical study of 11 cases. *Am J Clin Pathol* 2000; **113**: 345-350.
27. La Rosa S, Sessa F, Uccella S. Mixed Neuroendocrine-Nonneuroendocrine Neoplasms (MiNENs): Unifying the Concept of a Heterogeneous Group of Neoplasms. *Endocr Pathol* 2016; **27**: 284-311.
28. Lloyd R, Osamura R, Klöppel G, *et al.* WHO Classification of Tumours of Endocrine Organs. ed). IARC: Lyon, 2017.
29. Klimstra DS. Pathologic Classification of Neuroendocrine Neoplasms. *Hematol Oncol Clin North Am* 2016; **30**: 1-19.
30. Klimstra DS, Beltran H, Lilenbaum R, *et al.* The spectrum of neuroendocrine tumors: histologic classification, unique features and areas of overlap. *Am Soc Clin Oncol Educ Book* 2015: 92-103.
31. Yang Z, Tang LH, Klimstra DS. Gastroenteropancreatic neuroendocrine neoplasms: historical context and current issues. *Semin Diagn Pathol* 2013; **30**: 186-196.
32. Rietman EA, Platig J, Tuszynski JA, *et al.* Thermodynamic measures of cancer: Gibbs free energy and entropy of protein-protein interactions. *J Biol Phys* 2016.
33. Meder L, König K, Ozretic L, *et al.* NOTCH, ASCL1, p53 and RB alterations define an alternative pathway driving neuroendocrine and small cell lung carcinomas. *Int J Cancer* 2016; **138**: 927-938.

34. Wick MR, Scheithauer BW. Oat-cell carcinoma of the thymus. *Cancer* 1982; **49**: 1652-1657.
35. Jones MH, Virtanen C, Honjoh D, *et al*. Two prognostically significant subtypes of high-grade lung neuroendocrine tumours independent of small-cell and large-cell neuroendocrine carcinomas identified by gene expression profiles. *Lancet* 2004; **363**: 775-781.
36. Aldape K, Zadeh G, Mansouri S, *et al*. Glioblastoma: pathology, molecular mechanisms and markers. *Acta Neuropathol* 2015; **129**: 829-848.
37. Ohgaki H, Burger P, Kleihues P. Definition of primary and secondary glioblastoma--response. *Clin Cancer Res* 2014; **20**: 2013.
38. Fusco N, Geyer FC, De Filippo MR, *et al*. Genetic events in the progression of adenoid cystic carcinoma of the breast to high-grade triple-negative breast cancer. *Mod Pathol* 2016; **29**: 1292-1305.
39. Asamura H, Kameya T, Matsuno Y, *et al*. Neuroendocrine neoplasms of the lung: a prognostic spectrum. *J Clin Oncol* 2006; **24**: 70-76.
40. Righi L, Volante M, Tavaglione V, *et al*. Somatostatin receptor tissue distribution in lung neuroendocrine tumours: a clinicopathologic and immunohistochemical study of 218 'clinically aggressive' cases. *Ann Oncol* 2010; **21**: 548-555.
41. Travis WD, Rush W, Flieder DB, *et al*. Survival analysis of 200 pulmonary neuroendocrine tumors with clarification of criteria for atypical carcinoid and its separation from typical carcinoid. *Am J Surg Pathol* 1998; **22**: 934-944.
42. Nicholson SA, Beasley MB, Brambilla E, *et al*. Small cell lung carcinoma (SCLC): a clinicopathologic study of 100 cases with surgical specimens. *Am J Surg Pathol* 2002; **26**: 1184-1197.
43. den Bakker MA, Thunnissen FB. Neuroendocrine tumours--challenges in the diagnosis and classification of pulmonary neuroendocrine tumours. *J Clin Pathol* 2013; **66**: 862-869.
44. den Bakker MA, Willemsen S, Grunberg K, *et al*. Small cell carcinoma of the lung and large cell neuroendocrine carcinoma interobserver variability. *Histopathology* 2010; **56**: 356-363.
45. Sequist LV, Waltman BA, Dias-Santagata D, *et al*. Genotypic and histological evolution of lung cancers acquiring resistance to EGFR inhibitors. *Sci Transl Med* 2011; **3**: 75ra26.